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Heterocyclic *ortho*-quinones, a novel type of Photosystem II inhibitors

Walter Oettmeier [a,*], Klaus Masson [a], Hans-Jürgen Hecht [b]

[a] *Lehrstuhl Biochemie der Pflanzen, Ruhr-Universität, D-44780 Bochum, Germany*
[b] *Gesellschaft für Biotechnologische Forschung, Abt. Strukturforschung, Mascheroder Weg 1, D-31824 Braunschweig, Germany*

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Abstract

Members of the new chemical class of 7-substituted 6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-diones were found to be excellent inhibitors at the Q_B site of the photosystem II D1 reaction center protein. The best inhibitors with pI_{50}-values of >7 are: dimethyl-propyl, 7.05; *i*-pentyl, 7.36; *t*. butyl, 7.47; and *i*-propyl, 7.51. Displacement experiments with [^{14}C]atrazine revealed that the 8,9-diones behave non-competitively in respect of Photosystem II herbicides and, hence, have to be considered as a new type of Photosystem II inhibitors. This notion is further corroborated by their inhibitory activity in D1 mutants of *Chlamydomonas reinhardtii*.

Keywords: 7-Substituted 6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-dione; Dichlorophenol–indophenol reduction; [^{14}C]Atrazine displacement; Q_B binding site

1. Introduction

In the light reaction of Photosystem II an electron is transferred from a special chlorophyll molecule via pheophytin to the primary quinone acceptor Q_A and then to the secondary quinone acceptor Q_B. Plastoquinone, the resident in the Q_B binding pocket in this process, is double-reduced and accepts two protons. The resulting plastoquinol is released and replaced by a new plastoquinone from the pool [1]. It has been known since a long time that inhibitors of Photosystem II can compete with the native plastoquinone for binding because of their higher affinity to the Q_B-site and, hence, block Photosystem II electron transport. Some of these inhibitors are commercially used as herbicides (for review, see [2]). Photosystem II herbicides can belong to a variety of different chemical classes, for instance triazines, triazinones, ureas, biscarbamates and phenols, to cite only a few [2].

Quinone type inhibitors of Photosystem II are worthy of special interest because their structure is closely related to the natural electron acceptor plastoquinone. We have reported on the inhibition of Photosystem II electron transport by mostly halogen-substituted benzo- [3–7], naphtho- [7,8] and anthraquinones [9]. Here we wish to show that a new class of heterocyclic *ortho*-quinones (Fig. 1A) are highly potent inhibitors of Photosystem II at the Q_B-site. To ascertain the general structure of the *ortho*-quinones, an X-ray analysis of the best *ortho*-quinone type inhibitor was performed.

* Corresponding author. Fax: +49-234-321-4322;
E-mail: walter.oettmeier@ruhr-uni-bochum.de

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2. Materials and methods

2.1. Chemical synthesis

The 6-bromo-7-alky-benzo[4,5]imidazo[1,2α]pyridine-8,9-diones (Fig. 1A) were prepared in a three-step synthesis by radical alkylation of 1,4-benzoquinone [10], exhaustive bromination of the alkyl-1,4-benzoquinone and ring closure with 2-amino-pyridine. As an example, the synthesis of 6-bromo-7-*i*-propyl-benzo[4,5]imidazo[1,2α]pyridine-8,9-dione is shown below. The 6-bromo-7-alkyloxy-benzo[4,5]-imidazo[1,2α]pyridine-8,9-diones were prepared by nucleophilic substitution of tetrabromo-1,4-benzoquinone with the corresponding alcohol and subsequent ring closure with 2-amino-pyridine as described below. For 6,7-dibromo-benzo[4,5]imidazo-[1,2α]pyridine-8,9-dione, tetrabromo-1,4-benzoquinone was directly reacted with 2-amino-pyridine.

2.1.1. 2-i-propyl-1,4-benzoquinone [10]

To a mixture of 5.4 g (50 mmol) freshly sublimed 1,4-benzoquinone and 6.6 g (75 mmol) isobutyric acid in a solution of 1.3 g $AgNO_3$ (7.5 mmol) in 100 ml H_2O are added at 60–65°C under vigorous stirring a solution of 11.4 g (50 mmol) of ammonium persulfate in 50 ml H_2O during a period of 45 min. Stirring is continued for another 10 min and after cooling the solution is extracted with CH_2Cl_2, neutralized with Na_2CO_3, dried and the solvent evaporated in vacuo. Recrystallized from CH_3OH/H_2O, yield 4.5 g (73%), Fp. 34°C.

2.1.2. 2,3,5-Tribromo-6-i-propyl-1,4-benzoquinone

4.5 g (32 mmol) 2-*i*-propyl-1,4-benzoquinone, 20 g (125 mmol) Br_2 and 15 g (180 mmol) Na-acetate in 200 ml acetic acid are stirred at 65°C for 15 h and then an additional 4 h at 110°C (DC-control). The mixture is poured into H_2O and for removal of HBr, air is bubbled through. The residue is filtered off, dried and recrystallized twice from *n*-hexane. Yield 9.4 g (78%), Fp. 52–53°C.

2.1.3. 6-Bromo-7-i-propyl-benzo[4,5]imidazo[1,2α]-pyridine-8,9-dione

1.1 g (3 mmol) 2,3,5-tribromo-6-*i*-propyl-1,4-benzoquinone and 0.28 g (3 mmol) 2-amino-pyridine in 30 ml EtOH are refluxed for 4 h, than additional 0.28 g (3 mmol) of 2-amino-pyridine are added. The mixture is refluxed overnight, dried, extracted with hot H_2O, the residue is filtered, dried and either flash chromatographed on Kieselgel 60 (Fluka) with petrolether (60–90°C)/ethyl acetate (9:1, by volume) or recrystallized from benzene. Yield 0.33 g (35%), Fp. 222°C.

2.2. X-ray crystallography

Crystal lattice data for 6-brom-7-*i*-propyl-benzo[4,5]imidazo[1,2α]pyridine-8,9-dione ($C_{14}H_{11}BrN_2O_2$, $M_r = 319.16$ g/mol) were determined by precise angle measurement of 27 reflections using a Siemens P4 diffractometer and Cu–K_a radiation ($l = 1.54178$ Å) as triclinic P-1, $Z = 2$, $a = 5.59(1)$, $b = 8.94(1)$, $c = 13.321(2)$ Å, $a = 72.75(1)$, $\beta = 88.42(1)$, $g = 86.54(2)$, $V = 639.4(2)$, $r_{(calc)} = 1.658$ g/cm^3. Data collection was carried out by the omega scan technique ($2° < q < 57°$). Three standard reflections were monitored periodically and used for correction of crystal decay (< 10%). Of the 1687 measured reflections 1675 had $I > 2s$. The structure was solved by direct methods and refined on F^2 for all reflections with positive F^2 using SHELXTL (Siemens). Hydrogen atoms were generated with SHELXTL after anisotropic refinement of the non-hydrogen atoms. They were included in the full-matrix least-squares refinement restrained to the corresponding non-hydrogen atom and with isotropic temperature factors. The resulting *R*-value for 176 variables and 1687 observations was $R = 0.0943$, based on F and using $w = 1/[\s^2(Fo^2)+(0.1860P)^2+0.6251P]$ with $P = (Fo^2+2Fc^2)/3$ as weighting scheme.

2.3. Isolation and growth of wild-type and D1 mutants of Chlamydomonas reinhardtii

Wild-type and D1 mutants of *Chlamydomonas reinhardtii* (MZ2, $Ala_{251} \rightarrow Val$; Ar207+, $Phe_{255} \rightarrow Tyr$; MZ1, $Ser_{264} \rightarrow Ala$; and MZ4, $Leu_{275} \rightarrow Phe$) were isolated and grown as described recently [11].

2.4. Determination of pI_{50}-values

Chloroplasts from spinach and *C. reinhardtii* were prepared according to [12] or [11], respectively. Uncoupled electron flow from water to 2,6-dichlorophe-

nol-indophenol (DCIP) was measured spectrophotometrically at 600 nm in a Zeiss PMQII spectrophotometer, equipped for cross illumination with actinic light. Assays contained 20 μg chlorophyll in 30 mM Hepes–NaOH (pH 7.0), 10 mM $MgCl_2$, 3 μg gramicidin, 1 μM 2′,4,4′-trinitro-2′-iodo-3′-methyl-6′-isopropyl-diphenylether (DNP-INT) and 60 μM DCIP in a total volume of 3 ml. DNP-INT was included to prevent DCIP-reduction by Photosystem I [13]. The pI_{50}-value is the negative decadic logarithm of the concentration which inhibits DCIP-reduction by 50%. The basal rate of DCIP-reduction was 490 μmol DCIP (mg Chl)$^{-1}$ h^{-1}.

2.5. Displacement of [^{14}C]atrazine

For displacement experiments, chloroplasts from spinach corresponding to 100 μg chlorophyll were suspended in 2 ml of a medium containing 20 mM Tricin/NaOH-buffer (pH 8.0) and 20 mM $MgCl_2$. [^{14}C]Atrazine (CIBA-Geigy; spec. act. 210 MBq/mmol) was added as methanolic solution. The concentration of methanol never exceeded 1%. Samples were incubated for 5 min and then the *o*-quinone was added and the sample incubated for another 5 min. Chloroplasts were removed by centrifugation at 22 000×*g* for 10 min. The pellet was resuspended in 1 ml of buffer and pellet and supernatant assayed for radioactivity with Zinnser Quicksafe A in a LKB 1219 Rackbeta liquid scintillation counter. Counts were corrected for quenching.

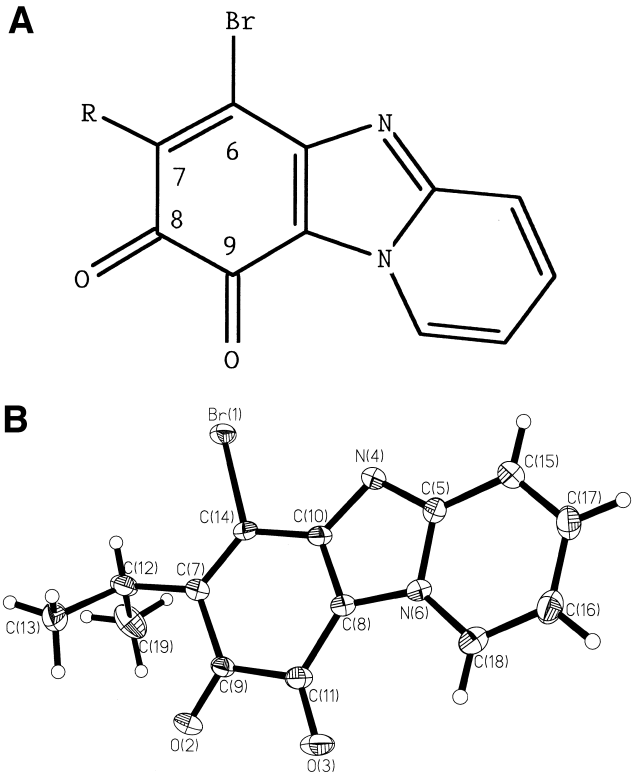

Fig. 1. (A) Structural formula of 7-substituted 6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-diones. (B) Plot of the crystal structure of 6-brom-7-*i*-propyl-benzol[4,5]imidazo[1,2α]pyridine-8,9-dione showing the non-hydrogen atoms as 75% probability thermal ellipsoids. The crystallographic data have been deposited as 'supplementary publication co. CCDC 151416' at the Cambridge Crystallographic Data Centre. Copies can be obtained at the address: CCDC, 12 Union Road, Cambridge CB21EZ (Fax: +44-1223-336-033; E-mail: deposit@ccdc.cam.ac.uk).

3. Results and discussion

Three-membered heterocyclic *o*-quinones with two nitrogens are not known in the literature as yet. In order to establish their chemical nature, a X-ray analysis of the most potent Photosystem II inhibitor, 6-bromo-7-*i*-propyl-benzo[4,5]imidazo[1,2α]pyridine-8,9-dione (No. 21, Table 1) was performed which proved the assumed structure without doubt (Fig. 1B). It should be noted that the corresponding four-membered heterocyclic *o*-quinone, the 6b,11-diazabenzo[α]fluorene-5,6-dione has been obtained by reaction of 2,3-dichloro-1,4-naphthoquinone with 2-amino-pyridine. Originally, they were thought to be *p*-quinones [14], but their correct structure was revised 6 years later [15]. In the latter paper a detailed mechanism for the formation of the *o*-quinone is also discussed. 6b,11-Diazabenzo[α]fluorene-5,6-dione is a weak Photosystem II inhibitor and exhibits a pI_{50}-value of ∼4 (not shown).

Table 1 lists the pI_{50}-values of 21 different 7-substituted 6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-diones in Photosystem II-mediated DCIP-reduction. The pI_{50}-values are in the range from 5.19 (compound 1, Table 1) to 7.51 (compound 21, Table 1) indicating that the 8,9-diones are indeed powerful inhibitors of Photosystem II. As is evident from Table 1, substitution in the 7-position by a *n*-alkyl- or *n*-alkyloxy-moiety renders inhibitors which are medium active (compounds 2, 3, 5–7, 9–13, 15, Table

Table 1
pI_{50}-values[a] of various 7-substituted 6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-diones in Photosystem II

No.	Code	*R*	pI_{50}
1	HCQ45	$(CH_3)_3C–CH_2$-	5.19
2	HCQ53	*n*-propyloxy-	5.52
3	HCQ60	2, 2, 2-trifluoroethyl-	5.62
4	HCQ37	phenyl-	5.72
5	HCQ54	*n*-butyloxy-	5.77
6	HCQ58	ethoxy-	5.92
7	HCQ50	*n*-butyl-	6.33
8	HCQ69	Br-	6.36
9	HCQ33	*n*-pentyl-	6.40
10	HCQ49	*n*-hexyl-	6.44
11	HCQ34	methyl-	6.62
12	HCQ43	*n*-propyl-	6.66
13	HCQ42	ethyl-	6.70
14	HCQ32	$(CH_3)_2CH–CH_2$-	6.76
15	HCQ48	*n*-heptyl-	6.80
16	HCQ47	$(C_3H_7)_2CH$-	6.80
17	HCQ38	cyclohexyl-	6.90
18	HCQ41	$C_2H_5–C(CH_3)_2$-	7.05
19	HCQ40	$(C_2H_5)_2CH$-	7.36
20	HCQ36	*t*.-butyl-	7.47
21	HCQ35	*i*-propyl-	7.51

[a]pI_{50}-values in Photosystem II were determined using the artificial electron acceptor 2,6-dichlorophenol-indophenol and as donor the native water splitting system (for conditions, see Section 2).

1) and their pI_{50}-values are in the range from 5.52 to 6.80. To the same class belong 7-phenyl- (compound 4, Table 1, pI_{50}-value 5.72) and 7-bromo-8,9-dione (compound 8, Table 1, pI_{50}-value 6.36). In general, the *O*-alkyl-compounds are less active then the alkyl derivatives; compare 2 and 12 ($\Delta pI_{50} = 1.14$), 6 and 13 ($\Delta pI_{50} = 0.78$) and 5 and 7 ($\Delta pI_{50} = 0.56$). In addition, fluorine substitution of the alkyl side chain also lowers activity (compare 13 and 3, $\Delta pI_{50} = 1.08$). However, the pI_{50}-value increases, if a branched alkyl side chain is situated in position 7 (compounds 16–21, Table 1). Compounds 18–21 (Table 1) exhibit pI_{50}-values of >7 and, thus, are in the range of commercially used herbicides like atrazine, terbutryn, metribuzin and phenmedipham, to cite only a few [2]. It should be noted, however, that the branched alkyl side chain should be directly attached to the carbon atom of the 8,9-dione moiety. If an additional methylene group separates the branched alkyl side chain from the 8,9-dione, the activity dramatically drops (compare 1 and 20, $\Delta pI_{50} = 2.28$, and 14 and 21, $\Delta pI_{50} = 0.75$, Table 1).

By comparison of 1,4-benzo- and 1,4-naphthoquinones as inhibitors of Photosystem II to 7-substituted 6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-diones, some common structural features can be recognized. As in the *t*.-butyl- and *i*-propyl-6-bromo-8,9-diones (compounds 20 and 21, Table 1) these substitutions can also be found in the inhibitory active 1,4-benzoquinones (-1,4-benzoquinone, pI_{50}-value): 2-bromo-3,4-di-*i*-propyl, 6.66; 2,6-dibromo-3-methyl-5-*i*-propyl, 7.11; 2,6-dibromo-3,5-di-*i*-propyl, 7.15; 2-bromo-5-*t*.-butyl, 6.18; 2,3-dibromo-5-*t*.-butyl, 7.24 [3]. Obviously, the *i*-propyl- and *t*.-butyl-group are optimally suited as a lipophilic anchor for tight binding in the Q_B niche. The situation is similar in the naphthoquinone series (-1,4-naphthoquinone, pI_{50}-value): 2-bromo-*n*-butyl, 5.71; 2-bromo-*i*-propyl, 5.73; 2-bromo-*n*-heptyl; 5.74 [8]. On

Table 2
pI_{50}-values of various 7-substituted 6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-diones in wild-type and D1 mutants of *Chlamydomonas reinhardtii*

No.	Code	Wild type	MZ2 $Ala_{251} \rightarrow Val$	Ar207+ $Phe_{255} \rightarrow Tyr$	MZ1 $Ser_{264} \rightarrow Ala$	MZ4 $Leu_{275} \rightarrow Phe$
12	HCQ43	6.40	6.68	6.70	6.26	6.70
13	HCQ42	6.05	6.12	6.13	5.92	6.49
14	HCQ32	6.15	6.95	6.57	6.36	6.77
15	HCQ48	6.53	6.26	7.30	6.60	6.62
16	HCQ47	6.15	5.32	6.30	6.00	5.82
17	HCQ38	6.38	6.60	7.28	6.22	6.92
18	HCQ41	6.92	6.61	6.66	7.26	6.92
19	HCQ40	7.02	6.30	7.10	6.85	7.38
20	HCQ36	6.98	6.53	6.96	6.85	7.24
21	HCQ35	7.10	7.00	7.23	7.00	7.44

the contrary, in 9,10-anthraquinones, inhibitory activity is achieved by the presence of halogen and hydroxy groups in the anthraquinone moiety [9].

In order to get more insight into the binding behaviour of 7-substituted 6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-diones within the Q_B binding niche with respect to other Photosystem II herbicides, the displacement of [^{14}C]atrazine by the 7-*n*-heptyl-8,9-dione (compound 15, Table 1) has been investigated. It is known since a long time that the 'classical' Photosystem II herbicides, like atrazine, metribuzin, phenmedipham, terbutryn and diuron displace themselves competitively from the thylakoid membrane, indicating an identical, common binding site [16,17]. Furthermore, the displacement between the 'classical' herbicides and phenolic herbicides (ioxynil, dinoseb, 2-iodo-4-nitro-6-isobutylphenol) also proceeds competitively, though both types of herbicides differ largely in their properties (for review, see [18]). From the inhibitory activity of herbicides in herbicide resistant mutants of cyanobacteria, algae and higher plants [19], Trebst has concluded that the 'classical' herbicides orient themselves preferentially towards Ser_{264} of the Photosystem II D1 protein, whereas the binding of phenolic herbicides occurs via His_{215} [20]. Nevertheless, as already stressed, 'classical' and phenolic herbicides exhibit competitive displacement behaviour.

A double-reciprocal plot of the binding of [^{14}C]atrazine in the presence of various concentrations of 7-*n*-heptyl-8,9-dione is shown in Fig. 2. As is evident from Fig. 2, the intercept of the regression lines lies on the abscissa, which indicates noncompetitive displacement [21]. Hence, the heterocyclic *o*-quinones differ in their binding behaviour from the other Photosystem II herbicides.

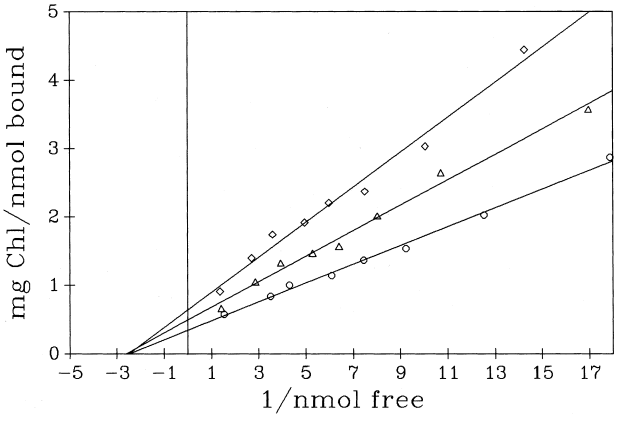

Fig. 2. Double-reciprocal plot of the binding of [^{14}C]atrazine in the presence of (○) 0, (△) 10, and (◇) 60 nmol of 7-*n*-heptyl-6-bromo-benzo[4,5]imidazo[1,2α]pyridin-8,9-dione.

This notion is further corroborated by the analysis of the pI_{50}-values of selected *o*-quinones in various D1 mutants of *C. reinhardtii* in comparison to the wild type. As can be seen from Table 2, the pI_{50}-values of all *o*-quinones in the mutants are in the same range as those of the wild type; the maximal difference is 0.9 (No. 17, Ar207+). This is in sharp contrast to the 'classical' herbicides, which exhibit a high degree of resistance in these mutants. For instance, mutants MZ1 and MZ2 are highly resistant towards atrazine, metamitron and metribuzin, which for metribuzin may be up to 4 orders of magnitude [19]. Mutant Ar207+ is supersensitive against triazinones [19]. Resistance against diuron is observed in mutants MZ1, MZ2 and MZ4 [19]. These few examples out of more (see [19]) emphasize the unique properties of *o*-quinones as inhibitors of Photosystem II.

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